

Inflammation promotes stomach epithelial defense by stimulating the secretion of antimicrobial peptides in the mucus

Megi Vllahu, Antonia Voli, Valerio Licursi, Claudia Zagami, Antonella D'Amore, Jan Traulsen, Sara Woelffling, Monika Schmid, Robbie Crickley, Richard Lisle, Alexander Link, Alessandra Tosco, Thomas F. Meyer & Francesco Boccellato

To cite this article: Megi Vllahu, Antonia Voli, Valerio Licursi, Claudia Zagami, Antonella D'Amore, Jan Traulsen, Sara Woelffling, Monika Schmid, Robbie Crickley, Richard Lisle, Alexander Link, Alessandra Tosco, Thomas F. Meyer & Francesco Boccellato (2024) Inflammation promotes stomach epithelial defense by stimulating the secretion of antimicrobial peptides in the mucus, Gut Microbes, 16:1, 2390680, DOI: [10.1080/19490976.2024.2390680](https://doi.org/10.1080/19490976.2024.2390680)

To link to this article: <https://doi.org/10.1080/19490976.2024.2390680>



© 2024 The Author(s). Published with license by Taylor & Francis Group, LLC.



[View supplementary material](#)



Published online: 08 Sep 2024.



[Submit your article to this journal](#)



Article views: 773

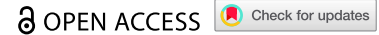


[View related articles](#)



[View Crossmark data](#)

RESEARCH PAPER



Inflammation promotes stomach epithelial defense by stimulating the secretion of antimicrobial peptides in the mucus

Megi Vllahu^{a,b*}, Antonia Voli^{id a,c*}, Valerio Licursi^{id d*}, Claudia Zagami^{id c}, Antonella D'Amore^{id c}, Jan Traulsen^{id c}, Sara Woelffling^b, Monika Schmid^b, Robbie Crickley^c, Richard Lisle^c, Alexander Link^{id e}, Alessandra Tosco^a, Thomas F. Meyer^{id b,f}, and Francesco Boccellato^{id b,c}

^aDepartment of Pharmacy, University of Salerno, Fisciano, Salerno, Italy; ^bDepartment of Molecular Biology, Max Planck Institute for Infection Biology, Berlin, Germany; ^cLudwig Institute for Cancer Research, Nuffield Department of Clinical Medicine, University of Oxford, Oxford, UK; ^dInstitute of Molecular Biology and Pathology (IBPM), National Research Council (CNR) of Italy c/o Department of Biology and Biotechnology "C. Darwin", Sapienza University, Rome, Italy; ^eDepartment of Gastroenterology, Hepatology and Infectious Diseases, Otto-von-Guericke University Hospital, Magdeburg, Germany; ^fLaboratory of Infection Oncology, Institute of Clinical Molecular Biology, Christian Albrecht University of Kiel and University Hospital Schleswig-Holstein - Campus Kiel, Kiel, Germany

ABSTRACT

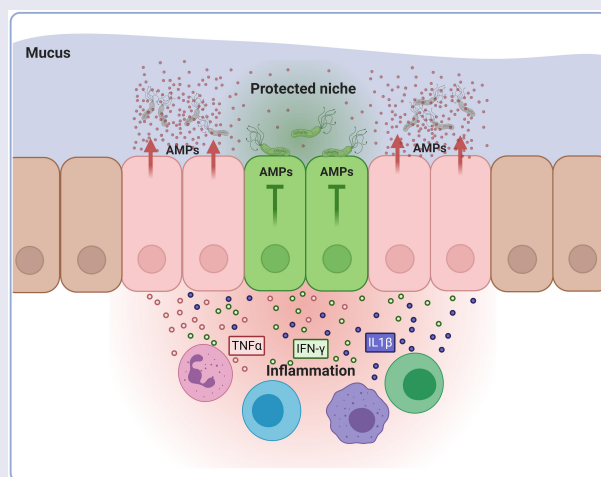
The mucus serves as a protective barrier in the gastrointestinal tract against microbial attacks. While its role extends beyond merely being a physical barrier, the extent of its active bactericidal properties remains unclear, and the mechanisms regulating these properties are not yet understood. We propose that inflammation induces epithelial cells to secrete antimicrobial peptides, transforming mucus into an active bactericidal agent. To investigate the properties of mucus, we previously developed mucosoid culture models that mimic the healthy human stomach epithelium. Similar to organoids, mucosoids are stem cell-driven cultures; however, the cells are cultivated on transwells at air-liquid interface. The epithelial cells of mucosoids form a polarized monolayer, allowing differentiation into all stomach lineages, including mucus-secreting cells. This setup facilitates the secretion and accumulation of mucus on the apical side of the mucosoids, enabling analysis of its bactericidal effects and protein composition, including antimicrobial peptides. Our findings show that TNF α , IL1 β , and IFN γ induce the secretion of antimicrobials such as lactotransferrin, lipocalin2, complement component 3, and CXCL9 into the mucus. This antimicrobial-enriched mucus can partially eliminate *Helicobacter pylori*, a key stomach pathogen. The bactericidal activity depends on the concentration of each antimicrobial and their gene expression is higher in patients with inflammation and *H. pylori*-associated chronic gastritis. However, we also find that *H. pylori* infection can reduce the expression of antimicrobial encoding genes promoted by inflammation. These findings suggest that controlling antimicrobial secretion in the mucus is a critical component of epithelial immunity. However, pathogens like *H. pylori* can overcome these defenses and survive in the mucosa.

ARTICLE HISTORY

Received 1 July 2024
Revised 30 July 2024
Accepted 5 August 2024


KEYWORDS

Mucus; Inflammation; *Helicobacter pylori*; Antimicrobial peptides; Stomach; Innate immunity; Epithelial defence



CONTACT Francesco Boccellato  francesco.boccellato@ludwig.ox.ac.uk  Ludwig Institute for Cancer Research, Nuffield Department of Clinical Medicine, University of Oxford, Oxford OX37DQ, UK

*These authors contributed equally.

 Supplemental data for this article can be accessed online at <https://doi.org/10.1080/19490976.2024.2390680>

© 2024 The Author(s). Published with license by Taylor & Francis Group, LLC.

This is an Open Access article distributed under the terms of the Creative Commons Attribution License (<http://creativecommons.org/licenses/by/4.0/>), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited. The terms on which this article has been published allow the posting of the Accepted Manuscript in a repository by the author(s) or with their consent.

Introduction

The gastrointestinal epithelium is a dynamic barrier that separates the host from potentially harmful microbes ingested with food. This barrier is maintained through a balance of physical structures (glands, crypts), immune cells, and secreted factors, including mucus and antimicrobial-peptides (AMPs).^{1,2} Mucus is a complex mixture of glycoproteins and other molecules which provides a physical barrier³ and a unique environment for the interaction with microorganisms.⁴ Experiments in animal models suggest that microbial control in the gut takes place in the mucus, as antimicrobial activity is restricted to the mucosal environment and does not extend to the gut lumen.⁵ AMPs are small proteins that play a crucial role in the innate immune response across various organisms, including mammals, insects, plants, and bacteria.^{6,7} These polypeptides are known for their broad-spectrum antimicrobial activity against bacteria, fungi, viruses, and even some parasites.⁸ AMPs are usually amphipathic: their hydrophobic residues mediate insertion into bacterial membranes, while clusters of positive residues facilitate membrane disruption and bacterial death.⁹ Within the mucosa, AMPs such as defensins and cathelicidins play a vital role in controlling microbial populations and maintaining homeostasis.¹⁰ Their expression usually correlates to infections or inflammation of the mucosa.

Helicobacter pylori is a Gram-negative bacterium with a unique tropism for the human stomach, where it is the only known colonizer.¹¹ Infection is associated with various gastrointestinal diseases, including ulcers and gastric cancer.¹² This unique host–pathogen interaction provides an opportunity to understand the epithelial host response to infection, set against the backdrop of the complex interactions of microbial communities that are typically found in the lower part of the gut. In infected human stomachs it has been reported the expression of AMPs such as human-beta-defensins^{13,14} and Cathelicidins such as LL-37¹⁵, while in infected mouse models it has been reported the expression of intellectin-1.¹⁶

However, there is little direct evidence of how the epithelium regulates secretion of AMPs into the mucus. Moreover, the overall efficacy of mucus in defending against specific pathogens has not been

measured. This limitation is mainly due to the lack of *in vitro* models of cells producing native mucus to directly investigate the secreted AMPs and their bactericidal capacity. We have previously developed a stem cell driven model of the healthy human stomach called “mucosoid cultures”.¹⁷ Similarly to organoids, the epithelial cells of the mucosoids can regenerate indefinitely by preserving their stem cell niches.¹⁷ Using different cultivation cocktails, these unique cultures display features of the human gastric epithelium *in-situ* including secretion of pepsin, gastric acid, and hormones.^{17,18} Importantly, cells in these cultures can be induced to differentiate into mucus-producing cells and accumulate mucus on the apical side.^{17,18} This unique feature makes mucosoids an excellent model in which to study the impact of this poorly accessible body fluid in controlling microbial invasion. Herein, we take advantage of the unique capabilities of mucosoid cultures to investigate the secretion of AMPs into the mucus during inflammation, and the interplay of this with *H. pylori*.

Material and methods

Human stomach

The epithelial cells used in this study to generate mucosoids are derived from weight loss surgery conducted at the Centre for Obesity and Metabolic Medicine, Helios Klinikum, Berlin-Buch, Germany (Table 1). These procedures were carried out with the approval of the ethics committee of Charité University Hospital, Berlin (EA1/129/12). Prior to their inclusion in the study, each participant provided written informed consent. Anonymized tissue samples were collected from patients, with a body mass index (BMI) greater than 36 kg/m². To prepare the samples, the tissue was first rinsed in phosphate-buffered saline (PBS) containing 50 µg/mL gentamicin in order to remove any blood. Subsequently, excess fat and connective tissue were carefully removed with a scalpel. From each sample, a 2 × 2 cm² section was excised from the central part of the antrum for further analysis.

Table 1. Weight loss surgery list of patients.

Code	Date of isolation	Age	Gender	Comments
GAT17	2015-07-14	30	Female	BMI: 53, <i>H.pylori</i> unknown status
GAT23	2016-04-15	55	Female	BMI:45, <i>H.pylori</i> negative
GAT27	2017-05-10	36	Female	BMI:69, <i>H.pylori</i> negative, Diabetes II
GAT28	2017-05-16	32	Male	BMI: 43, <i>H.pylori</i> negative
GAT29	2017-05-16	43	Female	BMI: 48, <i>H.pylori</i> negative
GAT31	2017-04-12	53	Female	BMI:58, <i>H.pylori</i> negative

The gastric biopsies used in this study (Figure 4) were collected from patients undergoing upper gastrointestinal endoscopy at the Department of Gastroenterology, Hepatology and Infectious Diseases at the Otto-von-Guericke University Magdeburg, Germany (Table 2). The study protocol was approved by the Institutional Review Board of Otto-von-Guericke University Magdeburg (ethics reference 80/11). The detailed study design was described previously.¹⁹ Briefly, every participant provided written informed consent before study inclusion. A pair of biopsies was immediately snap-frozen in liquid nitrogen and stored at -80°C until further processing and extraction of nucleic acids. Additional biopsies from the gastric antrum, the incisura angularis, and the gastric corpus were processed for routine histopathology assessment according to the updated Sydney classification.²⁰ This includes grading of the activity of the mucosal

inflammation by assessment of the infiltration by neutrophil granulocytes and the chronicity of inflammation by assessment of the infiltration by lymphocytes accordingly. According to the updated Sydney system, *Helicobacter pylori* density was assessed by histopathology. Status of *H. pylori* infection was determined at the clinic by rapid urease test and microbiology (culture), as well as serology (anti *H.pylori* IgM and IgG titer). Patients were defined as *H. pylori*-positive with positive microbiology and/or positive histology and/or positive serology. Positive serology only indicates past rather than active infection. Patients negative in these modalities were defined as *H. pylori*-negative. In case of inflammation but no structural changes of the mucosa, the sample was classified as Chronic gastritis (CG). In case of no inflammation, no structural changes and no *H.pylori* infection, the sample was classified as normal (N).

Table 2. Clinical biopsies data.

ID	Sex	Age [yrs]	Group	<i>H. pylori</i> status	Side of the stomach	Pathology Biopsy				Grading	
						Activity	Chronicity	Atrophy	IM	OLGA	OLGIM
33	m	20	N	0	Corpus	0	0	0	0	0	0
40	f	65	N	0	Corpus	0	1	0	0	0	0
48	f	88	N	0	Corpus	0	0	0	0	0	0
55	m	61	N	0	Corpus	0	0	0	0	0	0
56	f	31	N	0	Corpus	0	0	0	0	0	0
68	m	52	N	0	Corpus	0	0	0	0	0	0
56	f	31	N	0	Antrum	0	1	0	0	0	0
60	f	40	N	0	Antrum	0	1	0	0	0	0
27	f	60	CG	2	Corpus	0	2	0	0	0	0
61	f	51	CG	2	Corpus	1	2	0	0	0	II
98	f	19	CG	2	Corpus	1	1	0	0	0	0
99	m	42	CG	2	Corpus	2	2	0	0	0	0
111	f	46	CG	2	Corpus	2	2	0	0	0	0
121	f	51	CG	2	Corpus	2	2	0	0	0	I
122	f	56	CG	2	Corpus	1	1	0	0	0	0
27	f	60	CG	2	Antrum	0	2	0	0	0	0
99	m	42	CG	2	Antrum	1	2	0	0	0	0
108	f	35	CG	2	Antrum	2	2	0	0	0	0
110	f	47	CG	2	Antrum	2	2	0	0	0	0
122	f	56	CG	2	Antrum	1	2	0	0	0	0
126	f	70	CG	2	Antrum	1	1	0	0	0	0
128	f	33	CG	2	Antrum	1	2	0	0	0	0
129	m	54	CG	2	Antrum	2	2	0	0	0	0
141	m	47	CG	2	Antrum	0	2	0	0	0	0

Abbreviations and coding: f = female, m = male; Group: AG = atrophic gastritis, CG = chronic gastritis without atrophy, N = normal without inflammation; *H. pylori* status: 0 = negative, 1 = serology positive, 2 = serology and direct proof by either histology, microbiology or rapid urease test. See main text for scoring of the other parameters as well as OLGA/OLGIM staging.

Generation and maintenance of the mucosoid cultures

Gastric tissue from sleeve resections was processed as described previously. The tissue (0.5–1 cm²) from antrum and from corpus was cut into pieces <1 mm² and washed in cold PBS until the supernatant was clear (8–10 times), followed by a 30-min incubation in chelating solution (5.6 mM Na₂HPO₄; 8.0 mM KH₂PO₄; 96.2 mM NaCl; 1.6 mM KCl; 43.4 mM sucrose; 54.9 mM d-sorbitol; 0.5 mM dl-dithiothreitol; 2 mM EDTA in H₂O) at 37°C on a shaking platform. Settled tissue fragments were transferred to a Petri dish and subjected to gentle pressure under a glass slide to extract the glands. The extracted portions of tissue were re-suspended in medium containing 10% heat-inactivated fetal calf serum (Biocrom) to reduce cell re-aggregation. After settling the larger fragment for 1 min, the solution with the isolated glands was transferred into a new tube, centrifuged at 250xg for 5 min and re-suspended in 600 µL of Matrigel (356231; Corning), and aliquoted in 50 µL drops in each well of a 24-well plate. The cell aggregates were cultivated to form organoids, according to a protocol published previously.²¹ The organoids were cultivated for two passages before seeding them into mucosoids using a method established previously.¹⁷ Briefly, 250,000 cells derived from antrum organoids were seeded in 200 µL culture medium into collagen-coated (A10644–0, 12.5–15 µg/cm²; Gibco) Transwell inserts (PIHP01250; Millipore) placed in a 24-well plate at 37°C, 5% CO₂ in a humidified incubator. The space between filter and well was filled with 400 µL culture medium (Table 3). At day 3 post seeding, the medium

overlying the cells was removed from the well insert to start the air–liquid interface culture. Subsequently, the 500 µL medium below the filter was replaced twice a week. After 13 days, only half of the medium volume was changed regularly to maintain cell-secreted factors in the medium. Every 30–45 days, the mucosoids were expanded; top and bottom of inserts were washed three times with PBS, followed by 30–60 min of incubation with 0.05% trypsin/EDTA (25300; Thermo Scientific) on both sides of the filter. Cells were harvested, washed, and reseeded at 250,000 cells per new filter.

Microarray analysis

Microarray experiments were carried out as single-color hybridizations using custom whole genome human 8 × 60k Agilent arrays (Design ID 048908), in accordance with the manufacturer's instructions. Probe intensities were extracted using Agilent Feature Extraction software. The resulting single-color raw data files were analyzed using R v.4.4.2 and Bioconductor package limma v.3.56.2²² applying background correction with method “normexp” with offset 15 and quantile normalization. Differential gene expression analysis was performed using lmFit and eBayes functions from limma package accounting for the batch effect due by patient interindividual differences adding a covariate for patients in the regression model. Benjamini-Hochberg adjusted and nominal p-values were generated for each probe. Comparisons of microarray gene expression between groups were conducted using paired tests under different conditions. R packages ggplot2

Table 3. Composition of the culture medium.

Name	Concentration	Manufacturer	Code
ADF	18,45% V/V	Thermo Fischer	12634
Conditioned Wnt3A-medium	50% V/V	Home made	
Conditioned R-spondin1 medium	25% V/V	Home made	
HEPES	10 mM	Thermo Fischer	15630-056
Glutamax	1% V/V	Thermo Fischer	35050-087
B27	2% V/V	Thermo Fischer	17504044
N2	1% V/V	Thermo Fischer	17502048
Human epidermal growth factor (EGF)	20 ng/ml	Thermo Fischer	PHG0311
Human noggin (Peprotech)	150 ng/ml	Peprotech	120-10C-1000
Human fibroblast growth factor (FGF)-10	150 ng/ml	Peprotech	100-26-1000
Nicotinamide	10 mM	Sigma	N0636
Numan gastrin	10 nM	Sigma	G9145
A83-01	1 µM	Calbiochem	616454
Y-27632 ^a	7,5 µM	Sigma	Y0503

Note: ^aafter the 3rd day the concentration is reduced to 1,5 µM.

v.3.5.0. and ComplexHeatmap v.2.16.0 were used to generate heatmaps. Broad Institute gene set enrichment analysis (GSEA)²³ was used to assess the enrichment of the ranked gene expression profiles versus the curated “Hallmark” and selected C2 (REACTOME subcategory) and C5 (Gene Ontology, Biological Process subcategory) gene set collections from the Broad Molecular Signatures Database (MSigDB) v.7.5.1. Normalized enrichment score (NES) was also calculated with GSEA, by considering differences in pathway size (i.e., gene set size) and allowing for comparisons between the gene sets. The microarray data have been made publicly available in the Gene Expression Omnibus (GEO; <https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE268591>) database of the National Center for Biotechnology Information. These data can be accessed using the GEO accession number: GSE268591.

Mass spectrometry sample preparation

The mucus samples were processed using the filter-aided sample preparation method²⁴, with modifications as described by Rodriguez-Pineiro et al.²⁵ Each sample was diluted in 200 μ L of 6 M guanidinium hydrochloride in 0.1 M Tris/HCl, pH 8.5 (GuHCl), and supplemented with 5 non-human proteins (10 pmol/protein) as internal standards. Cysteines were reduced by adding 30 μ L of 0.1 M dithiothreitol and incubating the mixture at 60°C for 20 min. The samples were then transferred to Microcon-30 kDa centrifugal filters (MRCF0R030) and washed with 200 μ L of GuHCl. Alkylation was carried out by adding 100 μ L of 0.05 M iodoacetamide and incubating the samples at room temperature for 20 min in the dark. This was followed by two washes with 100 μ L of GuHCl and two more with 100 μ L of 50 mM ammonium bicarbonate/5% acetonitrile. Proteins were digested overnight at 37°C with 0.2 μ g of sequencing-grade modified trypsin (V5111; Promega) in 40 μ L of 50 mM ammonium bicarbonate/5% acetonitrile. The resulting peptide mixtures were acidified with trifluoroacetic acid to a final concentration of 0.5% (vol/vol), desalted using ZipTip C18 (Millipore, 0.6- μ L bed volume), and then lyophilized.

Liquid chromatography with tandem Mass spectrometry analysis

The samples were analyzed using a QExact Plus mass spectrometer (Thermo Fisher Scientific) connected to a Dionex UltiMate 3000 RSLCnano System (Thermo Fisher Scientific). Each sample was resolubilized in 13 μ L of 2:98 (v/v) acetonitrile/water with 0.1% trifluoroacetic acid. Then, 10 μ L was loaded onto a C18 PepMap 100 trap column (300 μ m \times 5 mm; 5 μ m particle size, 100 Å pore size; Thermo Fisher Scientific). The flow rate was set at 20 μ L/min using 2:98 (v/v) acetonitrile/water with 0.1% trifluoroacetic acid for preconcentration and desalting. Separation occurred on an Acclaim C18 PepMap RSLC column (75 μ m \times 250 mm; 2- μ m particle size, 100-Å pore size; Thermo Fisher Scientific) at a flow rate of 300 nL/min. High-performance liquid chromatography solvent A (0.1% v/v formic acid in water) was used, and peptides were eluted with solvent B (80:20 v/v acetonitrile/water with 0.1% formic acid), starting from 3%, increasing to 40% over 85 min, and then to 98% in 5 min. The peptides were analyzed in a data-dependent acquisition mode, alternating between one mass spectrometry scan and ten tandem mass spectrometry scans for the most abundant precursor ions. The mass spectrometry scans covered a range of m/z 350–1600, with a resolution set to 70,000. Peptides were fragmented using higher-energy collisional dissociation at 27% normalized collision energy and measured in the orbitrap at a resolution of 17,500.

Proteins identification

Proteins were identified and quantified using MaxQuant software (version 1.6.26), searching the SwissProt human sequence database (released 2018_11, containing 20,412 entries). The search parameters included a maximum of two missed cleavages, with variable modifications of Oxidation (M), Acetyl (Protein N-term), pyro-Glu (Gln), and carbamidomethylation of cysteines as a fixed modification. The false discovery rate was set to 0.01 for proteins, peptides, and modified sites. A comprehensive list of proteins identified under each condition is provided in Supplementary File 1.

Western Blot analysis.

Mucus from mucosoids was collected and diluted with Laemmli buffer final concentration 2×. The samples were then sonicated and boiled for 10 min at 95°C. Proteins were separated by SDS-PAGE in 18% or 10% polyacrylamide gel according to the proteins molecular weight. After separation by electrophoresis, transfer was performed using Criterion blotter (Biorad). The Nitrocellulose membranes (Amersham Protran 0.45 µm) were stained 5 min with red ponceau (Sigma) and washed with Tris-buffered saline (TBS)-Tween 1X. After 1 h blocking with a 5% solution of fat-free milk in TBS-Tween 1X, the membranes were incubated with the following primary antibodies overnight at 4°C (Table 4). After 3 × 10 min washes, the membranes were developed using the corresponding secondary antibodies (Table 4). The antibody signal was detected with enhanced chemiluminescence substrate according to the manufacturer's instruction (Amersham ECL Select). Chemidoc MP imaging system (Biorad) was used for images acquisition.

RNA isolation and quantitative real-time polymerase chain reaction analysis

Filters containing the cells of the mucosoid cultures were excised from the inserts and total RNA was extracted and purified using the RNeasy Mini Kit (Qiagen, Hilden, Germany), according to the manufacturer's specification. The total RNA concentration was quantified using a NanoDrop spectrophotometer. For reverse transcription, the Tetro cDNA Synthesis Kit (Bioline) was employed. Quantitative real-time polymerase chain reaction (qPCR) was performed using the QuantStudio3 Real-Time PCR System (ThermoFisher) and the

SensiMix SYBR low-ROX Kit (Bioline). The specific primers used in this study are detailed in Table 5. Results from three independent experiments were analyzed using the Delta-Delta Ct method. HPRT1 was used as a human reference gene.

Cultivation of *H.pylori*

H.pylori-GFP is a P12 isogenic fluorescent strain, resistant to kanamycin. The cultivation was done in agar plates containing 36 g/L GC agar base (Remel; supplied by Oxoid, R453502) supplemented with 10% inactivated horse serum (Biochrom, S 9135), 1% nutrient mix (100 g/L D(+)Glucose, 10 g/L, L-Glutamin), 26 g/L, 100 mg/L vitamin B1, 20 mg/L Iron(III) nitrate ninhydrate, 3 ml/L Thiamine hydrochloride, 13 mg/L 4 aminobenzoic acid, 250 mg/L nicotinamide adenine dinucleotide, 10 mg/L Vitamin B12, 1.1 g/L L-Cysteine, 1 g/L Adenine hemisulfate, 30 mg/L Guanine Hydrochloride, 150 mg/L L-Arginine monohydrochloride, 500 mg/L Uracil, 0,48% HCL in water), 1 µg/mL Nystatin, 5 µg/mL Trimethoprim, 10 µg/mL Vancomycin and 8 mg/mL Kanamycin.

Bacterial survival assay in the mucus

Single epithelial cells from three different mucosoid lines were seeded onto new transwells for 15 days prior to starting the different treatments. Mucus was harvested after the treatments and centrifuged at 1700×g at room temperature for 4 min to remove debris. A suspension of 5 µL with 10⁵ CFU of a kanamycin resistant P12 isogenic *H. pylori* GFP strain was resuspended in 20 µL of the harvested mucus samples or in PBS as control and incubated for 120 min in a microcentrifuge tube at 37°C. 20 µL of four serial dilutions (10⁻¹ – 10⁻⁴) were plated on

Table 4. Antibodies.

Antibody	Host	Dilution IF	Dilution WB	Manufacturer	Code
C3a	Goat	1:100	1:1000	R&D systems	AF3677
CXCL9	Rabbit	1:100	1:1000	Cell signaling	303275
Lipocalin	Mouse	1:200	1:1000	Abcam	ab23477
Lactotransferrin	Mouse	1:100	1:200	Santa Cruz	sc -53,498
Anti-Mouse AF546	Donkey	1:500	N/A	Invitrogen	A10036
Anti-Rabbit AF488	Goat	1:500	N/A	Invitrogen	A11008
Anti-Goat AF647	Chicken	1:500	N/A	Invitrogen	A21449
Anti-Rabbit HRP	Goat	N/A	1:10000	Biorad	STAR208P
Anti-Mouse HRP	Goat	N/A	1:10000	Biorad	STAR208P
Anti-Goat HRP	Rabbit	N/A	1:1000	Biorad	1721034
Anti CagA – B300	Rabbit	1:100	N/A	Santa Cruz	sc -25,766
Anti CagA BK-20	Goat	1:100	N/A	Santa Cruz	sc -48,128

Table 5. Primers.

Gene		Sequence
HPRT1	Fw	5'-GAC CAG TCA ACA GGG ACA T-3'
	Rv	5'-CCT GAC CAA GGA AAG CAA AG-3'
IL8	Fw	5'-ACA CTG CGC CAA CAC AGA AAT-3'
	Rv	5'-ATT GCA TCT GGC AAC CCT ACA-3'
CXCL9	Fw	5'-ATT GGA GTG CAA GGA ACC CC-3'
	Rv	5'-GGG CTT GGG GCA AAT TGT TT-3'
LTF	Fw	5'-TGC TCC ACC AAC AGG CTA AA-3'
	Rv	5'-GCG ACA TAC TGT GGT CCC AA-3'
C3	Fw	5'-GCG AAT GGA CAA AGT CGG C-3'
	Rv	5'-CTC TGG GAA CTC ACT TCG GG-3'
NGAL	Fw	5'-ACT TCC AGG ACA ACC AAT TCC AG-3'
	Rv	5'-GGC AAC CTG GAA CAA AAG TCC-3'
TNF α	Fw	5'-TGA AAG CAT GAT CCG GGA CG-3'
	Rv	5'-CAG CTT GAG GGT TTG CTA CAA C-3'
IFN γ	Fw	5'-TCGGTAACTGACTTGAATGTCCA-3'
	Rv	5'-TCGCTTCCCTCTTTAGTGC-3'
IL-1 β	Fw	5'-ATGATGGCTTATTACAGTGGCAA-3'
	Rv	5'-GTCGGAGATTCTAGTGGGA-3'
rDNA 16s <i>H. pylori</i>	Fw	5'-TTTGTAGAGAAGATAATGACGGTATCTAAC-3'
	Rv	5'-CATAGGATTCACACCTGACTGACTAT C-3'
gDNA hGAPDH	Fw	5'-GACTTCAACAGCGACACC C-3'
	Rv	5'-AGAAGATGAAAAGAGTTGTCAGGGC-3'

supplemented GC agar plates. Plating of the suspension was performed by pouring the liquid on the edge and inclining the plate in one direction to spread the liquid in line without touching the skirt of the plate. Plates were incubated at 37°C, 5% CO₂ and 5% O₂, face-up for 1 h to let the excess liquid evaporate and then turned upside down for 3 days. Colonies were counted and results are presented as the percentage of colonies relative total colonies present in PBS controls (Figure 3(f)).

Bacterial survival assay in a solution with synthetic antimicrobials

10⁵ CFU of *H. pylori* P12-GFP (kanamycin resistant) were incubated for 60 and 120 min in a solution of 25 μ L of PBS containing 5, 10 or 30 μ g/ μ l of antimicrobial peptide (Table 6). Each AMP was tested individually, three times at each concentration. The suspension was plated in supplemented GC agar plates as it was done for the

bactericidal assay of the mucus. The results are presented as the percentage of CFU relative to the one in PBS.

Mucosoid treatment with cytokines and treatment with cytokines in combination with *H.pylori* infection

Three weeks post-seeding, 5 ng/mL TNF α , 2.5 ng/mL IL1 β and 10 ng/mL IFN γ were added individually or in combination in the culture medium of mucosoids for 5 days. In case of concomitant infection, 2 days after cytokines combination treatment, a suspension of 30 μ l of *H.pylori* (MOI 100) was added on the mucus of the mucosoids for the remaining 3 days (Figure 5(a)). To calculate the MOI, the number of cells (usually 600.000 to 700.000) was calculated from an identical mucosoid culture.

Table 6. Sequence of the AMPs.

hLFcin	GRRRRSVQWC ¹⁰ AVSQPEATKC ²⁰ FQWQRNMRKV ³⁰ RGPPVSCIKR ⁴⁰ DSPIQCIQA ⁵⁰
hLCN2	MPLGLLWLGL ¹⁰ ALLGALHAQA ²⁰ QDSTSDLIPA ³⁰ PPLSKVPLQQ ⁴⁰ NFQDNQFQGG ⁵⁰ WYVVLGAGNA ⁶⁰ ILREDKDPQK ⁷⁰ MYATIYELKE ⁸⁰ DKSYNVTSVL ⁹⁰ FRKKKCDYWI ¹⁰⁰ RTFVPGCQPG ¹¹⁰ EFTLGNIKSY ¹²⁰ PGLTSLYLRV ¹³⁰ VSTNYNQHAM ¹⁴⁰ VFFKKVSQNR ¹⁵⁰ EYFKITLYGR ¹⁶⁰ TKELTSELKE ¹⁷⁰ NFIRFSKSLG ¹⁸⁰ LPENHIVFPV ¹⁹⁰ PIDQCIDG
hC3a	SVQLTEKRMD ¹⁰ KVGKYPKELR ²⁰ KCCEDGMREN ³⁰ PMRFSCQRRR ⁴⁰ RFISLGEACK ⁵⁰ KVFLDCCNYI ⁶⁰ TELRRQHARA ⁷⁰ SHLGLAR
hCXCL9	TPVVRKGRCS ¹⁰ CISTNQGTIH ²⁰ LQSLKDLKQF ³⁰ APSPSCEKIE ⁴⁰ IATLKNQVQ ⁵⁰ TCLNPDADV ⁶⁰ KELIKKWEKQ ⁷⁰ VSQKKQKNG ⁸⁰ KKHQKKVVK ⁹⁰ VRKSQRSRQK KTT

Immunofluorescence staining of mucosoids

Mucosoids on the filters were fixed in 4% paraformaldehyde for 20 min at room temperature and washed with PBS. The filters were incubated with blocking solution (PBS, 5% donkey serum, 0.3% Triton X-100) for 1 h, followed by primary antibody in antibody dilution solution (PBS, 1% BSA + 0.3% triton X-100) overnight at 4°C. After three washes with PBS, samples were incubated with fluorescently labeled secondary antibodies and 4',6-diamidino-2-phenylindole (1:1000) for 2 h at room temperature in the dark. The antibodies and the relative dilutions are listed in Table 4. Samples were washed three times with PBS, mounted in Mowiol, and analyzed by confocal microscopy using a Zeiss 980 microscope. Images were processed and analyzed with ImageJ.

Software

Data analysis and plot generation were performed using R, MaxQuant, Microsoft Excel, and GraphPad Prism. Figures were assembled in Adobe Illustrator, with schematics created via Biorender.com. The manuscript was written in Microsoft Word and edited with the assistance of ChatGPT 4 to correct grammar and spelling.

Results

The effect of pro-inflammatory cytokines on gastric epithelial cells

Epithelial cells were isolated from the antrum of healthy stomachs derived from patients having undergone weight loss surgery (Table 1). Cells were cultivated on porous transwells at the air-liquid interface with a supplementation of growth factors to generate mucosoid cultures (Table 3).¹⁷ As previously observed, the inclusion of WNT3A, RSPO1, EGF and NOGGIN in the culture cocktail induced a mucus neck cells phenotype^{17,19,26} and the secretion of a MUC6 enriched mucus to the apical side.¹⁷ To study how epithelial cells respond to inflammatory stimuli, we analyzed the transcriptome of gastric mucosoids treated with TNF α , IL1 β and IFN γ in combination or single cytokine treatment. These cytokines are commonly found in the inflamed

gastric mucosa.^{27–29} Analysis of the genes upregulated in cytokine treated mucosoids revealed that pro-inflammatory pathways are significantly enriched (Figure 1(a)). We also find a positive correlation with genes encoding for AMPs (Figure 1(c)). Single treatment with TNF α and with IL1 β revealed a similar gene enrichment pattern and positive regulation of antimicrobial related genes (Supplementary SFig.1A–D). This similarity might be explained by the fact that both TNF α and IL1 β activate the NF- κ B pathway, a major effector of inflammation (Figure 1(d)). However, the combination of TNF α , IL1 β and IFN γ induces a stronger NF- κ B response (Figure 1(d) and Supplementary SFig.2A). Treatment with IFN γ specifically induced IFN γ target genes (Figure 1(e), Supplementary SFig.1E and Supplementary SFig.2B) but caused milder enrichment in antimicrobial peptide-associated genes compared to mucosoids treated with TNF α or IL1 β (Supplementary SFig.1F). Among all possible expressed AMPs in the mucosoids (Figure 1(f)), we found *LCN2* (Lipoprotein 2), *LTF* (lactotransferrin), *CXCL1* (C-X-C motif ligand 1), *C3* (complement component 3) and *BPIFA1* (BPI fold containing family A member 1) to be consistently up-regulated after treatment with TNF α , IL1 β and IFN γ . *REG3A* and *REG3G* (human regenerating islet-derived protein 3- α and γ), and *BPIFB1* (BPI fold containing family B member 1) expression was found to be dependent on IL1 β and TNF α . *CXCL9* (C-X-C motif ligand 1) was instead regulated only in the presence of IFN γ . Other antimicrobials previously reported in the stomach or in stomach cells^{14,30,31} such as defensins (e.g., DEFB4A, DEFB1, DEFB124, DEFB121 and DEFB103) or Cathelicidins (e.g., LL-37) did not undergo significant changes in their expression, or were undetectable, when the epithelium is inflamed. This suggests that other non-epithelial cells may be the source of these antimicrobials, or that their regulation does not depend on TNF α , IL1 β or IFN γ .

Inflammatory signals stimulate the epithelium to secrete antimicrobials in the mucus

To understand if the proinflammatory signals TNF α , IL1 β and IFN γ regulate epithelial secretions to the mucus, we analyzed by mass spectrometry the total protein content of the mucus accumulated

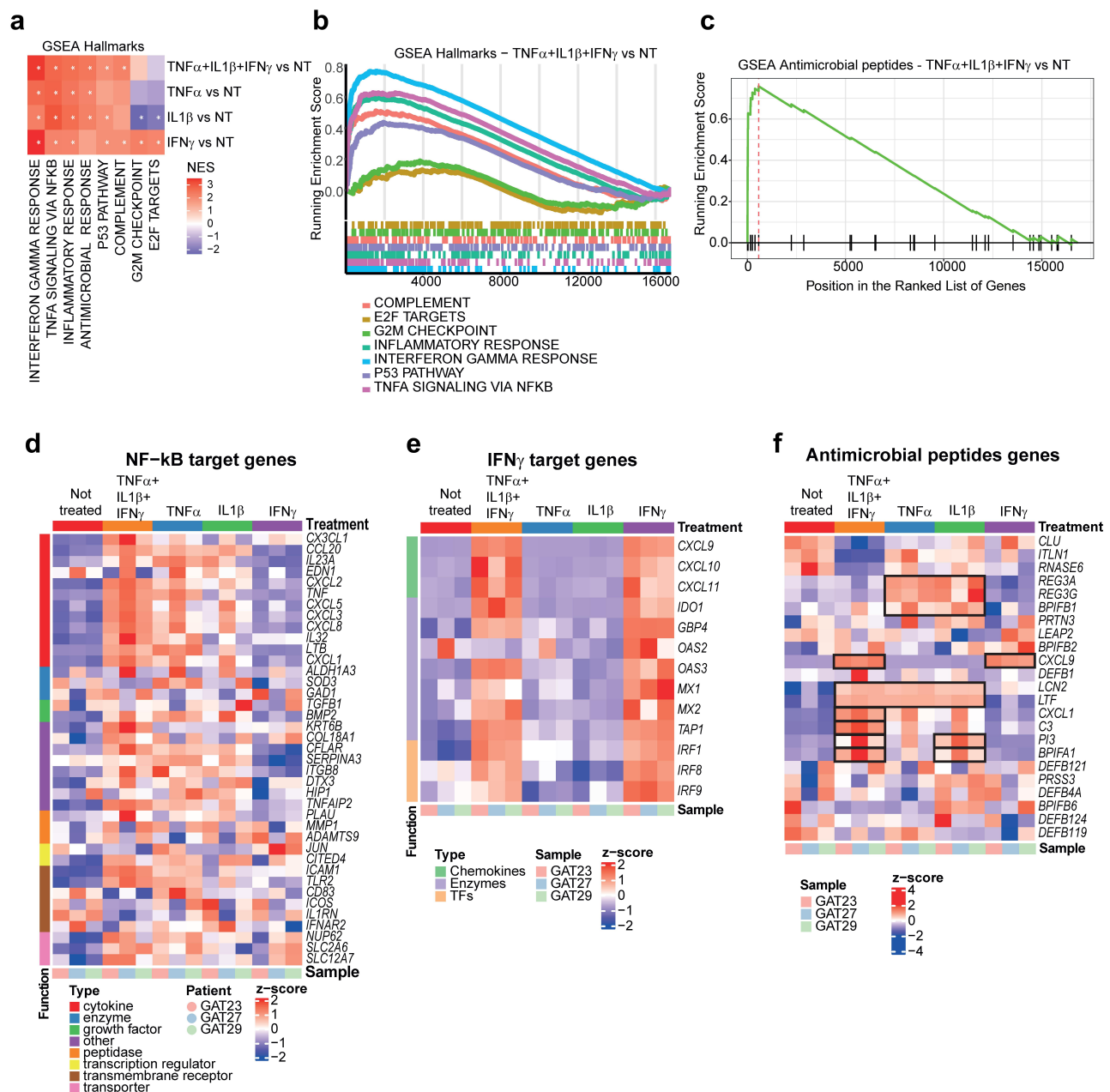


Figure 1. Inflammation triggers the expression of genes encoding for antimicrobial peptides. Cells from three different mucosoid lines were treated with TNF α (5 ng/mL), IL1 β (2.5 ng/mL), and IFN γ (10 ng/mL) combined or individually (as indicated), for 5 days before extracting RNA for transcriptomic profile by microarray. (a). Heatmap of the most relevant GSEA hallmarks (<https://www.gsea-msigdb.org/gsea/msigdb/collections.jsp>) relative to the comparison of the transcriptome of the treated vs. untreated mucosoids. NES= normalized enrichment score, *= false discovery rate < 0.0001. Red = upregulation, white = no change, blue = downregulation. (b). Enrichment plot of the most significant GSEA hallmark resulting from the comparison of the transcriptome of the mucosoids treated with TNF α , IL1 β , and IFN γ , compared to the untreated samples. (c). Enrichment plot of the GSEA hallmark “antimicrobial peptides” (d). Heatmap of validated nf- κ B target genes as reported in (<https://www.bu.edu/nf-kb/gene-resources/target-genes/>) (e). Heatmap of selected interferon-dependent chemokines, enzymes and transcription factors. (f). Heatmap of genes coding for antimicrobial peptides. Genes showing consistent upregulation in at least one of the conditions are outlined in black.

for five days (Figure 2(a)) in inflamed vs non inflamed mucosoids. We found numerous proteins in the mucus, such as mucins, proteases, enzymes, and cytokines, (Supplementary file 1) however,

only some of these were differentially expressed between inflamed and non-inflamed gastric epithelial cells (Figure 2(b) and Supplementary File 2). We also observed plasma membrane proteins as

well as proteins from other intracellular compartments, suggesting that intracellular content, probably resulting from dead cells, could be found in the mucus. Four known AMPs are significantly differentially accumulated in the mucus of the inflamed epithelium: Lactotransferrin (LTF), Lipocalin2 (LCN2), Complement component 3 (C3) and C-X-C motif chemokine 9 (CXCL9) (Figure 2(c–f)). LTF is an antimicrobial found in milk and other body secretions.³² LCN2 is a known major antimicrobial player in gut immunity.³³ C3 is a subunit of the complement system with antimicrobial activity.³⁴ CXCL9 is an antimicrobial whose activity is independent from the chemokine function of this peptide.³⁵

Other proteins encoded by AMP genes identified in the transcriptome analysis (Figure 1(f)) were either undetectable, or their variation in the mucus following inflammation in the epithelium was not statistically significant. The expression of LTF, LCN2, C3 and CXCL9, was confirmed by Western blot analysis of the mucus (Figure 2(g–k)) and their intracellular expression localized at the apical side of the mucosoids, consistent with the fact that these proteins are secreted in the mucus (Figure 2, (k–n)).

The efficacy of epithelial antimicrobials against *Helicobacter pylori*

To test if the AMPs found in the mucus were active against *H. pylori in vivo*, we incubated *H. pylori* with commercially available recombinant versions of the antimicrobial domains of these proteins at a range of concentrations (5–30 $\mu\text{g mL}^{-1}$) for 1 h and 2 h, and then selectively plated these solutions to assess bactericidal activity based upon the number of Colony Forming Units (CFUs) relative to a PBS control (Figure 3(a)). The 49 amino acid bioactive portion of human Lactotransferrin³⁶ displayed the greatest activity against *H. pylori*, with a 90% CFU reduction in 2 h using the concentration of 30 $\mu\text{g/mL}$ of the peptide (Figure 3(b)). Lipocalin2 showed a moderate bactericidal effect (20–50% of CFU reduction in 2 h) (Figure 3(c)). Moderate antimicrobial activity was also observed for the recombinant C3 and CXCL9 (Figure 3(d, e)) (40%–60% and 30%–50% of CFU reduction in 2 h, respectively). These results demonstrate that, individually, the AMPs found in the mucus exert an

antimicrobial activity against *H. pylori*. As these AMPs are secreted together in the mucus, we next investigated if native mucus from the inflamed epithelial cells of mucosoids shows antimicrobial activity. Mucosoids were treated again with TNF α , IL1 β and IFN γ in combination, and mucus was harvested after 5 days of accumulation in treated and untreated controls. *H. pylori* was able to form significantly fewer colonies after incubation with mucus from inflamed cells, compared to mucus from non-inflamed cells (Figure 3(f)). To test if this mucus protects against infection, we then treated mucosoids with TNF α , IL1 β and IFN γ for 5 days and concomitantly infected with *H. pylori* from day 2 to day 5 (Figure 3(g)). Our results demonstrate that there was a significantly higher number of remaining cells in the mucosoid cultures treated with pro-inflammatory cytokines compared to the non-treated (419 cells/ mm^2 vs. 2016 cells/ mm^2). This suggests that the mucus from inflamed cells conferred a partial protection against *H. pylori* infection.

AMPs gene expression is increased in chronic gastritis

Chronic gastritis is an inflammatory condition of the stomach often caused by chronic *H. pylori* infection.¹¹ Association with *H. pylori* infection can be made after the detection of the bacteria via urea breath test, histopathology report and *H. pylori* blood antibody test. As *Helicobacter pylori* can colonize deep within the glands^{37,38} where MUC6+ cells are located, we decided to test the hypothesis that AMPs found in inflamed mucosoids are upregulated in patients with *H. pylori*-related chronic gastritis. We analyzed 16 biopsies from *H. pylori*-positive chronic gastritis cases and 8 healthy unmatched controls from *H. pylori* negative patients without inflammation in the stomach (Table 2). We tested the expression of LTF, LCN2, C3 and CXCL9 by qPCR and found that these AMPs were significantly upregulated in the chronic gastritis group compared to healthy controls (Figure 4(a–d)). We also tested gene expression of the pro-inflammatory cytokines TNF α , IL1 β and IFN γ Figure 4(e–g), and correlated this to the expression of the AMPs (Figure 4(h)). TNF α showed the strongest linear correlation (R

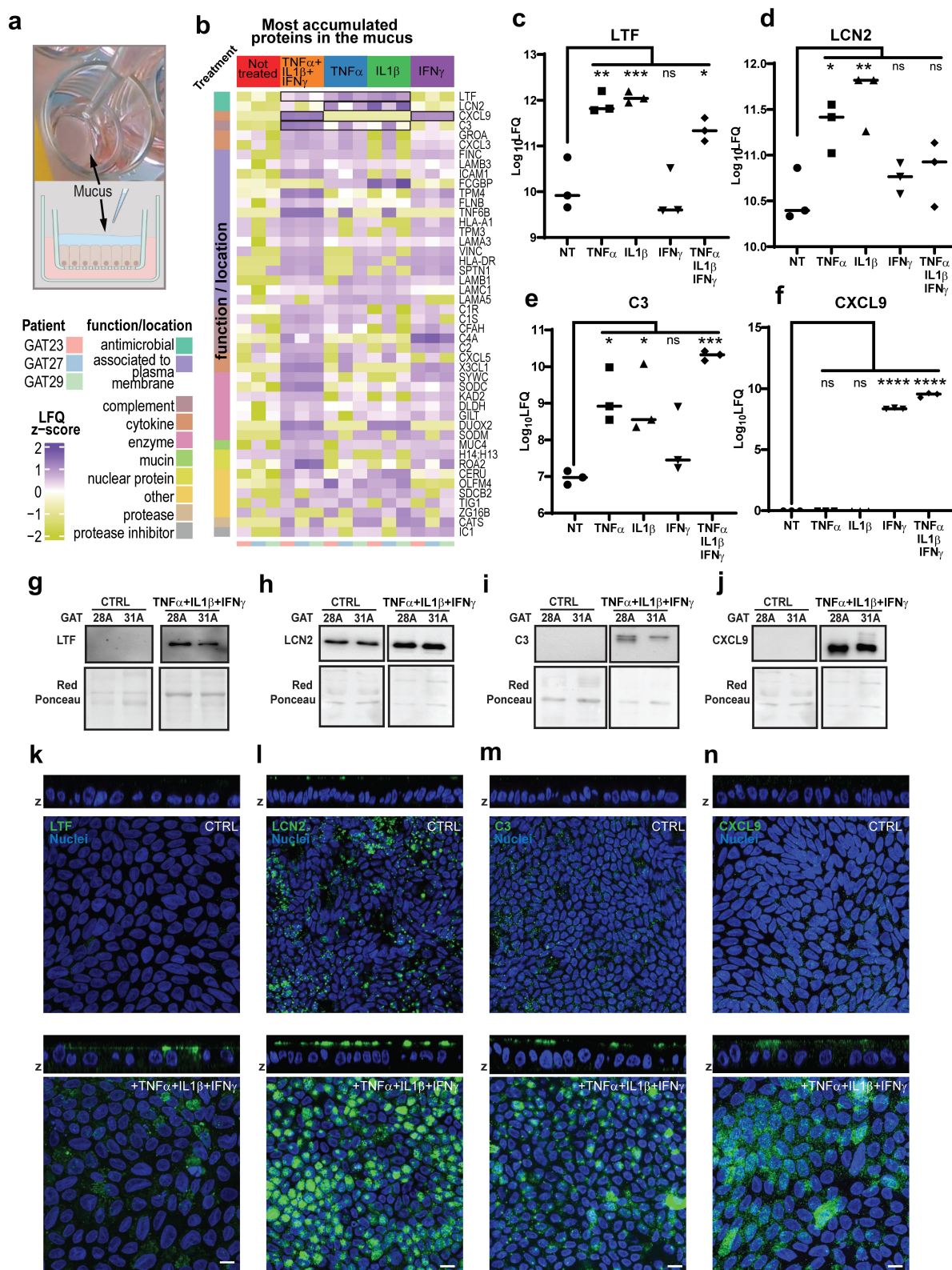


Figure 2. Inflammation induce the secretion of AMPs in the mucus. (a). Mucus was removed from three different mucosoid lines after treatment with TNF α (5 ng/mL), IL1 β (2.5 ng/mL), and IFN γ (10 ng/mL) combined or individually (as indicated) for 5 days. The total mucus secreted and accumulated was analyzed by mass spectrometry. (b). Heatmap of the abundance of the proteins in the mucus determined by label free quantification (LFQ). Only proteins with an altered expression of at least 10-fold in one of the conditions are represented. Outlined in black are those proteins with reported bactericidal activity. The other proteins are categorized by location or function. (c)(d)(e)(f) The Log₁₀LFQ values are plotted for LTF (lactotransferrin), LCN2 (lipocalin 2), C3 (complement component C3), CXCL9 (C-X-C motif chemokine ligand 9) the horizontal bar represent the median of tree individual measurements from the mucus

Pearson coefficient) with all four antimicrobials. *IL1B* poorly correlated, while *IFNG* correlated only with *CXCL9* (an interferon target gene). We also measured the relative amount of *H. pylori* DNA within the biopsies. As expected, there was a significant increase in *H. pylori* DNA in biopsies from patients with *H. pylori*-associated chronic gastritis (Supplementary SFig.3A). However, *H. pylori* DNA in biopsies did not correlate with the expression levels of genes encoding AMPs or those encoding pro-inflammatory cytokines (Supplementary Fig.3B). Altogether these results indicate that TNF α is likely to be the key factor for stimulating epithelial antimicrobial defense in the mucus.

Helicobacter pylori reduces the expression of epithelial antimicrobials

H. pylori has the exceptional capacity to persist within the stomach mucosa for decades if not eradicated with antibiotics.¹¹ While it is known that it can evade adaptive immunity, we now question how the bacteria survive given the presence of antimicrobials secreted by the inflamed epithelium. To investigate whether *H. pylori* is able to subvert host epithelial innate immune defense, we treated mucosoids with the same mixture of pro-inflammatory cytokines (TNF α IL1 β IFN γ) that triggered the secretion of LTF, LCN2, C3 and *CXCL9*, and then infected them with *H. pylori* (Figure 5(a)). The level of expression of IL8 (*CXCL8*) remained high in case of exposure to cytokines followed by infection (Figure 5(b)), suggesting that the level of inflammation remains constant. However, we noticed that in cytokine-treated mucosoids, the following infection with *H. pylori* caused a reduced expression of LTF, LCN2 and C3 (Figure 5(c-e)). *CXCL9* expression was not significantly

different whether *H. pylori* infection was present or not (Figure 5(f)). Interestingly, while LTF, LCN2 and C3 regulation depends on TNF α (Figure 2(c-e)) and correlates with *TNFA* expression in chronic gastritis patients (Figure 4(h)), *CXCL9* is the only antimicrobial dependent on IFN γ (Figure 2(f)). As TNF α promotes NF- κ B activation, *H. pylori* might mediate the downregulation of LTF, LCN2 and C3 by uncoupling TNF α or NF- κ B pathways activation from the induction of the expression of the antimicrobials.

Discussion

The gastrointestinal mucosa serves as a crucial barrier in the human body, fulfilling both digestive functions and defensive roles against microbial invasion. When infected, this mucosa becomes inflamed and attracts cells from both the innate and adaptive immune systems. The roles of various immune cells in defense are well-recognized, while the main contribution attributed so far to the epithelium is to produce mucus, which forms a physical barrier that prevents the attack of pathogens. Any further active defensive role of the epithelium remains to be demonstrated.

Mucus studies depend on the availability of this body fluid. Mucus can either be prepared directly from fixed animal or human tissue³⁹ or harvested from tissues mounted in perfusion chambers.^{25,40} These approaches, although capable of providing in-depth analysis of mucus under native conditions, have limited experimental settings for testing variations in mucus secretion. Alternatively, mucus-producing cell lines can be grown in polarized monolayers, a method commonly employed for airway

produced by three different mucosoid lines (GAT23, 27, 29). Multiple comparison test was performed after one way ANOVA to assess if the differences between treated and untreated samples are significant: * $p < 0.05$, ** $p < 0.005$, *** $p < 0.0005$, **** $p < 0.00005$. The relative abundance of other antimicrobials *CXCL1* and *CXCL3* did not pass the statistical test for any of the multiple comparisons. (g)(h)(i)(j) validation of expression of the antimicrobial was confirmed by Western blot using a further two samples of mucosoids (GAT28, GAT31) treated with the combination of pro-inflammatory cytokines as before. As housekeeping proteins are not yet available for the mucus, Red Ponceau was used as a loading control. (k)(l)(m)(n) Mucosoids were treated with a combination of pro-inflammatory cytokines as before, and immunofluorescence experiments carried out to localize the expression of intracellular LTF, LCN2, C3 and *CXCL9*. Stained whole mount mucosoids were imaged using confocal microscopy. Images were taken across the whole thickness of the monolayer (z-stacks) and the panels shows a top and a lateral projection. Scale bar = 5 μ m.

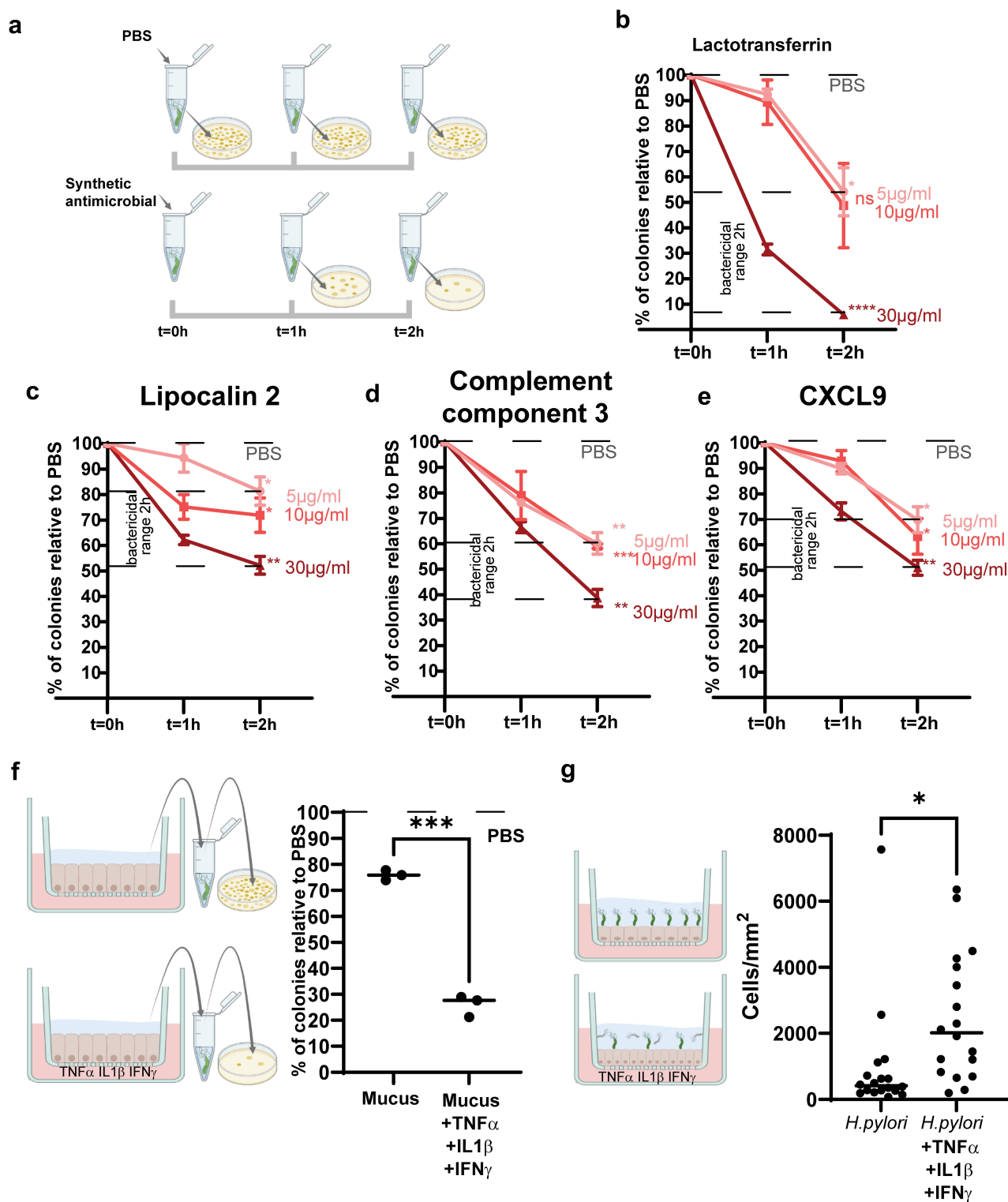


Figure 3. The bactericidal activity of antimicrobial peptides found in the mucus. ((a) A suspension of 10^5 CFU of kanamycin resistant *H. pylori* was incubated for 1 h and 2 h in the presence of different concentrations (5, 10, or 30 mg/mL) of AMPs. The suspension was plated in kanamycin plates and the number of colonies was counted relative to PBS to assess the bactericidal range of each antimicrobial. (b). Bactericidal activity of Lactotransferrin using the bioactive peptide lactoferrin (LFcin), the antimicrobial peptide derived from the hydrolysis of lactotransferrin. (c). Bactericidal activity of LCN2. (d). Bactericidal activity of C3a, the peptide derived from complement component 3. (e). Bactericidal activity of CXCL9. Multiple comparison test was performed after a two way ANOVA to assess if the difference in bactericidal activity, for each concentration of AMP, between different time points, is statistically significant. p values comparing $t = 2$ h and $t = 0$ are reported. ns= non significant. * $p < 0.05$, ** $p < 0.005$, *** $p < 0.0005$, **** $p < 0.00005$. (f). Mucosoids were treated with TNF α (5 ng/mL), IL1 β (2.5 ng/mL), and IFN γ (10 ng/mL) for 5 days, with a fresh supply of cytokines

epithelium mucus.⁴¹ However, as these cell lines often originate from cancerous tissues, their signaling pathways are altered, and their capacity to produce a robust mucus layer is generally reduced. Therefore, the regulation of protein secretion in the mucus remains poorly studied.

Bridging the gap between animal models and cell lines, organoid technology offers an unprecedented opportunity to explore mucus biology in depth.⁴² By transforming spherical organoids into mucosoid cultures we obtained the formation of an epithelial barrier that accumulates mucus on the apical side^{17,43} while retaining the stem cell-driven regeneration and multilineage differentiation capacity typical of the organoids. Mucosoid cultures, developed from healthy human stomach tissue, allow us to investigate for the first time how mucus secretion responds to various experimental conditions. In this study, we focused on antimicrobial peptides as they are recognized innate immune weapons against infection, known to be present in the mucus of various enteric^{5,44,45} and non-enteric mucosae.⁴⁶

We found that inflammation induces the stomach epithelium to secrete antimicrobial peptides in the mucus. Specifically, we observed that TNF α , IL1 β and IFN γ induce the secretion of four antimicrobials in the mucus: Lactotransferrin, Lipocalin2, complement component 3, and CXCL9. The mucus from inflamed cells and these specific antimicrobials have a significant anti-*H. pylori* activity *in-vitro* and the expression of these antimicrobials was also confirmed in biopsies from patients with inflammation in the stomach. Finally, we find that *H. pylori* counteracts the inflammation-induced expression of Lactotransferrin, Lipocalin2 and complement component 3.

Lactotransferrin (or Lactoferrin, LTF, TRLF, Lf) is an iron binding protein found at different concentrations (from 1–3 $\mu\text{g}/\text{mL}$ to 8 mg/mL) in

different body fluids including breast milk, colostrum, tears, and saliva.^{47–49} Its antimicrobial activity is independent from iron and is rather attributed to a peptide, called lactoferricin (Lfcin), derived from its hydrolysis or digestion with pepsin in the stomach. The antimicrobial properties of lactoferricin depend on the number of positively charged arginine residues located at the N-terminal domain.^{50,51} These residues can bind and disrupt the membranes of bacteria.⁵⁰ In some countries, lactotransferrin is a commercially available prebiotic derivation from cow's milk. However, while human Lfcin contains four arginine residues, the bovine variant has only two. This makes human Lfcin binding stronger to bacterial LPS compared to the bovine version.⁵¹ Notably, human lactotransferrin has demonstrated effectiveness in controlling *H. pylori* growth both *in vitro*⁵² and in animal models.⁵³ Here, we demonstrate that this antimicrobial is directly produced by inflamed human stomach epithelial cells.

Lipocalin2 (or NGAL) is a major player in gut immunity by maintaining bacterial populations far from the mucosa.³³ The bactericidal capacity depends on its ability to bind bacterial siderophores which prevent bacterial uptake of iron.⁵⁴ C3 is a subunit of the complement system with antimicrobial

activity. In particular, C3a is the peptide that results from the cleavage mediated by C3 convertase or by spontaneous hydrolysis of C3 in water. This peptide has a known antimicrobial activity from invertebrates to humans and it is also referred to as anaphylatoxin. The bactericidal activity depends on the capacity of this peptide to permeabilise the bacterial membrane.⁵⁵ CXCL9 is a chemokine but its chemokine-independent antimicrobial activity was reported against *Citrobacter rodentium* infection in the gut.^{34,56} The bactericidal mechanism is currently unknown. The different mode of action of these antimicrobials implies

added on the third day. Mucus samples from mucosoids derived from 3 different patients was incubated with 10^5 CFU of a kanamycin resistant isogenic P12 strain for 2 hours before plating and assessing the bactericidal activity by counting the percentage of colonies relative to PBS. Paired samples t-test was used to assess if the difference between the bactericidal activity of the two groups was significant. ***= $p < 0.0005$. (g) Mucosoids were treated with a combination of pro-inflammatory cytokines as before and infected on the second day with *H. pylori* at MOI 100 for 3 days. Mucosoids were washed with PBS to remove the dead cells, they were stained with DAPI and imaged using confocal microscopy to count the remaining cells after infection. Each dot represents the density of cells per area of imaging (0.18 mm^2) and paired samples t-test was used to assess if the difference between the groups is significant * $p < 0.05$.

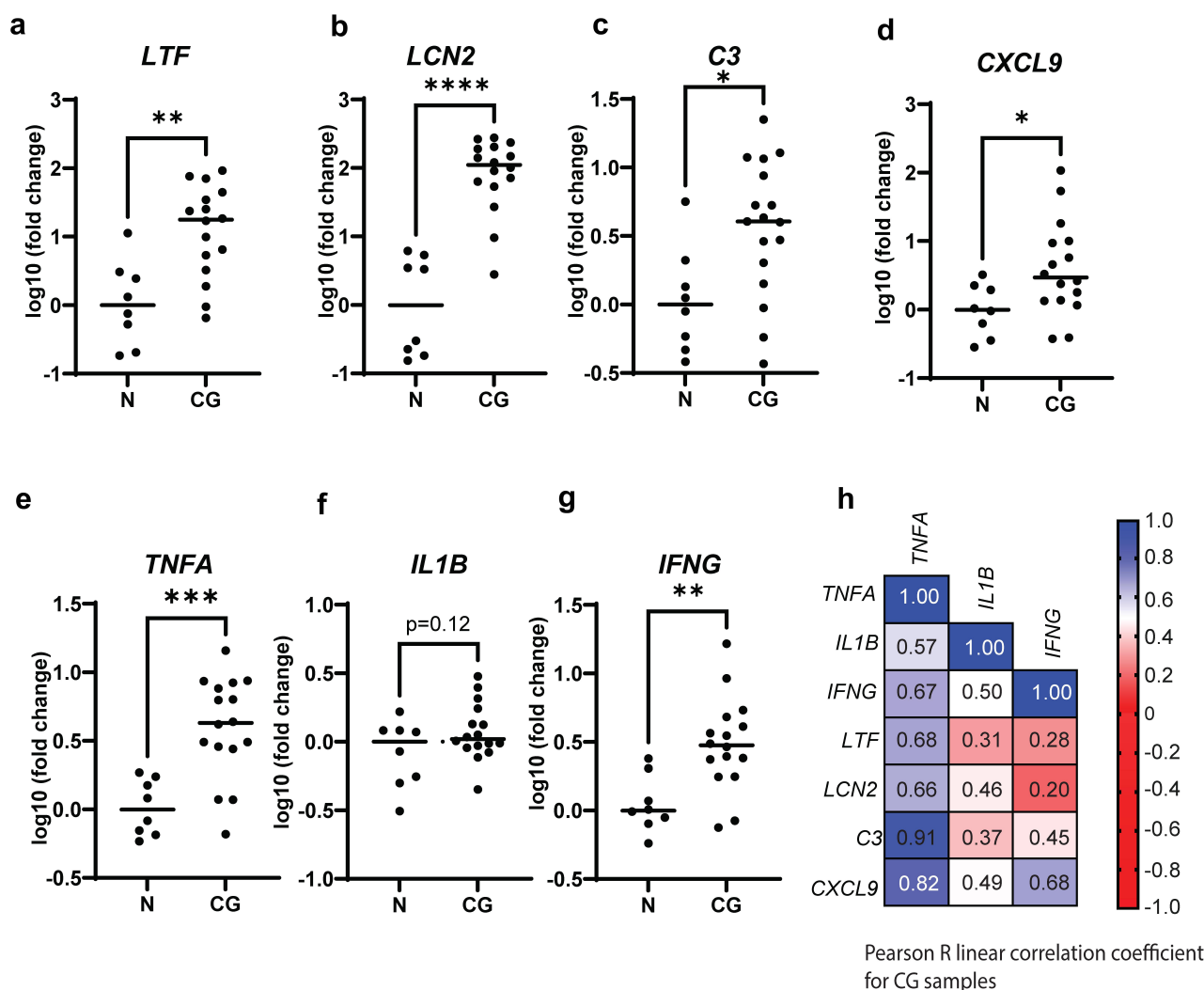


Figure 4. The expression of AMPs in biopsy samples from patients with chronic gastritis. RNA from 16 biopsy samples from *H. pylori* positive chronic gastritis (GC) cases and 8 from normal-looking stomach (N) was retrotranscribed into cDNA for the detection of AMP genes and of genes encoding for pro-inflammatory cytokines. (a)–(g). The Log₁₀ relative expression of *LTF*, *LCN2*, *C3*, *CXCL9*, *TNFA*, *IL1B* and *IFNG* is reported. The significance of the difference between the groups was calculated using an unpaired t-test. * $p < 0.05$, ** $p < 0.005$, *** $p < 0.0005$, **** $p < 0.00005$. (h). The Log₁₀ fold induction value of each gene detected in the chronic gastritis biopsies was used to compute a Pearson R linear correlation heatmap. 1 = highest correlation, 0 = no correlation, -1 = highest negative correlation.

that the human stomach epithelium has evolved multiple strategies to prevent bacterial colonization.

However, we also noted that while inflammation typically increases the expression of AMPs, the presence of *H. pylori* infection leads to a decrease in their expression. This observation suggests that *H. pylori* has developed mechanisms to circumvent epithelial innate defenses, aligning with its exceptional colonization ability. In the context of chronic infections, it can be hypothesized that if *H. pylori* rapidly colonizes a new gland before it is eliminated

by the mucus, the bacterium may neutralize the antimicrobial secretions of the epithelium, thereby facilitating the establishment of a new colony in a protected niche. Interestingly, *H. pylori* particularly reduces the expression of Lactotransferrin, Lipocalin2, and C3, which depend mainly on TNF α for their production. This indicates that *H. pylori* uncouples the pro-inflammatory signal of TNF α from its antimicrobial effectors. Interestingly both TNF α ⁵⁷ as well as *H. pylori*⁵⁸ can elicit an inflammatory response in epithelial cells through the activation of NF- κ B. However,

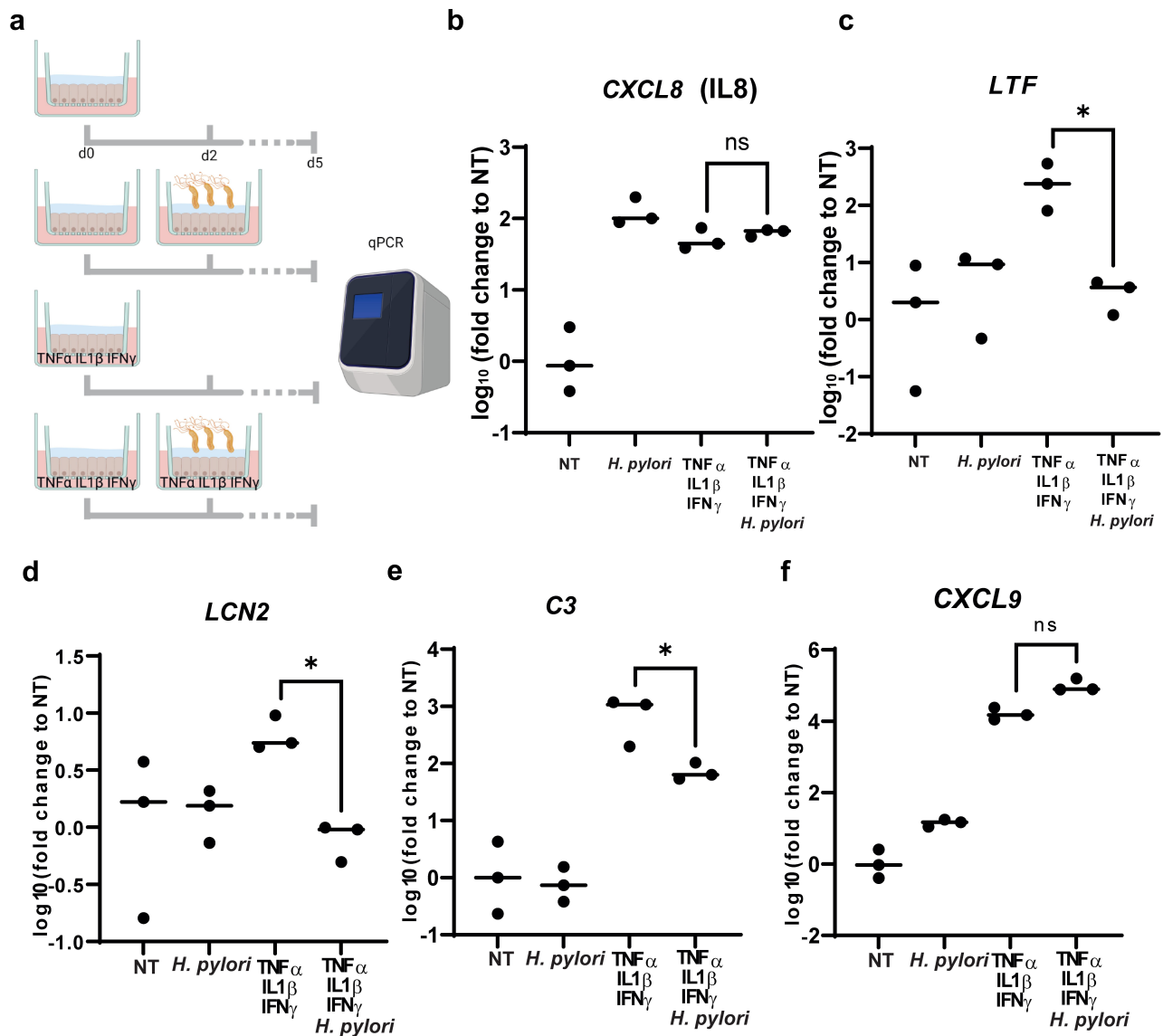


Figure 5. *H. pylori* downregulates the expression of antimicrobials of inflamed epithelial cells (a). Three different mucosoid lines were treated by four different protocols, according to the schematic. Upper: non-treated (NT); second row: *H. pylori* infection carried out at day 2 at MOI 100; third row: treatment with pro-inflammatory cytokines (TNF α , 5 ng/mL; IL1 β , 2.5 ng/mL; IFN γ 10 ng/mL) from day 0; lower: treatment with pro-inflammatory cytokines at the same concentrations as previously from day 0, following *H. pylori* infection at MOI 100 at day 2. At day 5, RNA was extracted and the abundance of transcripts was measured relative to non-treated controls. Due to the large differences between the expression of genes under different treatments, results are expressed in Log₁₀-fold change. (b). The expression of *CXCL8* does not change when *H. pylori* infection follows the treatment with pro-inflammatory cytokines. (c)(d)(e) The expression of *LTF*, *C3* and *LCN2* is downregulated when *H. pylori* infection is carried out in addition to the treatment with pro-inflammatory cytokines. (f) *CXCL9* expression does not significantly change when *H. pylori* infection is in addition to the treatment with pro-inflammatory cytokines. Multiple comparison test was performed after one way ANOVA to assess if the differences in gene expression between different treatments are significant. Only the result of the test comparing the samples treated with cytokines and the one infected and treated with cytokines are shown. ns = non significant. * $p < 0.05$.

the pathways of activation differ; *H. pylori* mediates p65 nuclear translocation via the activation of the kinase ALPK1.^{59–61} It is currently unknown whether the ALPK1-mediated activation of NF- κ B is synergistic with, or acts in opposition to, the TNF α pathway for specific target genes.

Moreover, the co-transcription factors involved in the expression of the antimicrobials found in this study are still unknown and merit further investigations. Understanding this dysregulation could reveal the key signaling molecules used by the bacterium and inform a potential pharmacological

approach to increase the secretion of these AMPs, providing support in eradicating the bacteria. Potential host-directed approaches to restore AMP expression in infected tissues could represent an attractive therapeutic avenue, especially in scenarios where antibiotics are becoming less effective.

Abbreviations

AMPs	anti microbial peptides
BPIFA1	BPI fold containing family A member 1
BPIFB1:	BPI fold containing family B member 1
BSA	bovine serum albumin
C3	complement component 3
cDNA	complementary deoxyribonucleic acid
CFU	colony forming unit
CG	chronic gastritis
CXCL1	C-X-C motif ligand 1
CXCL8	C-X-C motif ligand 8
CXCL9	C-X-C motif ligand 9
DEFB 4A,1,124,121,103	beta defensins 4A,1,124,121,103
ECL	Enhanced chemiluminescence
EDTA	Ethylenediaminetetraacetic acid
EGF	epidermal growth factor
GFP	gene set enrichment analysis
HPRT1	hypoxanthine phosphoribosyltransferase 1
IFN γ	interferon gamma
IgM, IgG	immunoglobulin M, G
IL1 β	interleukin 1 beta
LCN2	Lipopalin2
LL-37	cathelicidin antimicrobial peptide
LTF	lactotransferrin
MOI	multiplicity of infection
MUC6	mucin 6
NES	normalized enrichment score
NF- κ B	Nuclear factor kappa-light-chain-enhancer of activated B cells

PBS	phosphate buffered saline
qPCR	quantitative real-time polymerase chain reaction
REG3A,G	human regenerating islet-derived protein 3-alpha and gamma
RSLC	Rapid Separation Liquid Chromatography
RSPO1	R-spondin-1
SYBR	N',N'-dimethyl-N-[4-[(E)-(3-methyl-1,3-benzothiazol-2-ylidene)methyl]-1-phenylquinolin-1-ium-2-yl]-N-propylpropane-1,3-diamine
TBS	Tris-buffered saline
TNF α	Tumor necrosis factor Alpha
WNT3A	Wnt family member 3A

Acknowledgments

We extend our gratitude to Pau Morey for inspiring our mucus experiments, Dagmar Frahm for assistance with mucoid cultivation, and Hans Mollenkopf, Ina Wagner, and Hilmar Berger for their support with the microarrays. We are also grateful to Ravinash Krishna Kumar and Silja Weßler for commenting and improving this manuscript. This manuscript was professionally edited by Kate Dunning.

Disclosure statement

FB and TFM filed a patent concerning the mucoid cultures. No potential conflict of interest was reported by the other authors

Funding

The work was supported by the Ludwig Institute for Cancer Research, Max Planck Institute for Infection Biology and University of Salerno.

ORCID

Antonia Voli  <http://orcid.org/0009-0000-2410-8081>
 Valerio Licursi <http://orcid.org/0000-0002-8172-1437>
 Claudia Zagami  <http://orcid.org/0000-0001-5280-8261>
 Antonella D'Amore  <http://orcid.org/0000-0002-3441-4397>
 Jan Traulsen  <http://orcid.org/0000-0001-9340-9232>

Alexander Link  <http://orcid.org/0000-0002-9514-4562>
 Thomas F. Meyer  <http://orcid.org/0000-0002-6120-8679>
 Francesco Boccellato  <http://orcid.org/0000-0001-6013-7154>

Author contributions

Conceptualization: FB, TFM, AT.
 Methodology: MS, RC, RL, VL, SW, CZ, ADA
 Investigation: MV, AV, ADA, CZ, SW
 Provided clinical samples: AL
 Supervision: FB
 Writing original draft: FB

Data availability statement

Transcriptomic data have been made publicly available in the Gene Expression Omnibus (GEO; <https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE268591>) database of the National Center for Biotechnology Information. These data can be accessed using the GEO accession number: GSE268591.

References

- Turner JR. Intestinal mucosal barrier function in health and disease. *Nat Rev Immunol.* 2009;9(11):799–809. doi:10.1038/nri2653.
- Johansson ME, Hansson GC. Immunological aspects of intestinal mucus and mucins. *Nat Rev Immunol.* 2016;16(10):639–649. doi:10.1038/nri.2016.88.
- Hansson GC, Johansson ME. The inner of the two Muc2 mucin-dependent mucus layers in colon is devoid of bacteria. *Gut Microbes.* 2010;1(1):51–54. doi:10.4161/gmic.1.1.10470.
- Johansson ME, Sjövall H, Hansson GC. The gastrointestinal mucus system in health and disease. *Nat Rev Gastroenterol Hepatol.* 2013;10(6):352–361. doi:10.1038/nrgastro.2013.35.
- Meyer-Hoffert U, Hornef MW, Henriques-Normark B, Axelsson L-G, Midtvedt T, Putsep K, Andersson M. Secreted enteric antimicrobial activity localises to the mucus surface layer. *Gut.* 2008;57(6):764–771. doi:10.1136/gut.2007.141481.
- Hancock RE, Sahl HG. Antimicrobial and host-defense peptides as new anti-infective therapeutic strategies. *Nat Biotechnol.* 2006;24(12):1551–1557. doi:10.1038/nbt1267.
- Hancock RE, Haney EF, Gill EE. The immunology of host defence peptides: beyond antimicrobial activity. *Nat Rev Immunol.* 2016;16(5):321–334. doi:10.1038/nri.2016.29.
- Zaslhoff M. Antimicrobial peptides of multicellular organisms. *Nature.* 2002;415(6870):389–395. doi:10.1038/415389a.
- Shai Y. Mode of action of membrane active antimicrobial peptides. *Biopolymers.* 2002;66(4):236–248. doi:10.1002/bip.10260.
- Ganz T. Defensins: antimicrobial peptides of innate immunity. *Nat Rev Immunol.* 2003;3(9):710–720. doi:10.1038/nri1180.
- Malfertheiner P, Camargo MC, El-Omar E, Liou J-M, Peek R, Schulz C, Smith SI, Suerbaum S. Helicobacter pylori infection. *Nat Rev Disease Primers.* 2023;9(1). doi:10.1038/s41572-023-00431-8.
- Traulsen J, Zagami C, Daddi AA, Boccellato F. Molecular modelling of the gastric barrier response, from infection to carcinogenesis. *Best Pract Res Clin Gastroenterol.* 2021;50-51:101737. doi:10.1016/j.bpg.2021.101737.
- Bauer B, Wex T, Kuester D, Meyer T, Malfertheiner P. Differential expression of human beta defensin 2 and 3 in gastric mucosa of Helicobacter pylori-infected individuals. *Helicobacter.* 2013;18(1):6–12. doi:10.1111/hel.12000.
- Patel SR, Smith K, Letley DP, Cook KW, Memon AA, Ingram RJM, Staples E, Backert S, Zaitoun AM, Atherton JC, et al. Helicobacter pylori downregulates expression of human β -defensin 1 in the gastric mucosa in a type IV secretion-dependent fashion. *Cellular Microbiol.* 2013;15(12):2080–2092. doi:10.1111/cmi.12174.
- Hase K, Murakami M, Iimura M, Cole SP, Horibe Y, Ohtake T, Obonyo M, Gallo RL, Eckmann L, Kagnoff MF, et al. Expression of LL-37 by human gastric epithelial cells as a potential host defense mechanism against Helicobacter pylori. *Gastroenterology.* 2003;125(6):1613–1625. doi:10.1053/j.gastro.2003.08.028.
- Sigal M, Reinés MDM, Müllerke S, Fischer C, Kapalczyńska M, Berger H, Bakker ERM, Mollenkopf H-J, Rothenberg ME, Wiedenmann B, et al. R-spondin-3 induces secretory, antimicrobial Lgr5(+) cells in the stomach. *Nat Cell Biol.* 2019;21(7):812–823. doi:10.1038/s41556-019-0339-9.
- Boccellato F, Woelffling S, Imai-Matsushima A, Sanchez G, Goosmann C, Schmid M, Berger H, Morey P, Denecke C, Ordemann J, et al. Polarised epithelial monolayers of the gastric mucosa reveal insights into mucosal homeostasis and defence against infection. *Gut.* 2018;68(3):400–413. doi:10.1136/gutjnl-2017-314540.
- Woelffling S, Daddi AA, Imai-Matsushima A, Fritsche K, Goosmann C, Traulsen J, Lisle R, Schmid M, Reines-Benassar MDM, Pfannkuch L, et al. EGF and BMPs govern differentiation and patterning in human gastric glands. *Gastroenterology.* 2021;161(2):623–636.e616. doi:10.1053/j.gastro.2021.04.062.
- Link A, Langner C, Schirrmeister W, Habendorf W, Weigt J, Venerito M, Tammer I, Schlüter D, Schlaermann P, Meyer TF, et al. Helicobacter pylori vacA genotype is a predominant determinant of immune response to Helicobacter pylori CagA. *World*

- J Gastroenterol. 2017;23(26):4712–4723. doi:10.3748/wjg.v23.i26.4712.
20. Dixon MF, Genta RM, Yardley JH, Correa P. Classification and grading of gastritis. The updated Sydney system. International workshop on the histopathology of gastritis. In: Am J Surg Pathol. Vol. 20. Houston 1994. 1996. p. 1161–1181.
 21. Schlaermann P, Toelle B, Berger H, Schmidt SC, Glanemann M, Ordemann J, Bartfeld S, Mollenkopf HJ, Meyer TF. A novel human gastric primary cell culture system for modelling *Helicobacter pylori* infection in vitro. Gut. 2014;65(2):202–213. doi:10.1136/gutjnl-2014-307949.
 22. Ritchie ME, Phipson B, Wu D, Hu Y, Law CW, Shi W, Smyth GK. Limma powers differential expression analyses for rna-sequencing and microarray studies. Nucleic Acids Res. 2015;43(7):e47. doi:10.1093/nar/gkv007.
 23. Subramanian A, Tamayo P, Vamsi K, Mootha VK, Mukherjee S, Ebert BL, Michael A, Gillette MA, Paulovich A, Pomeroy SL, et al. Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles. Proceedings of the National Academy of Sciences of the United States of America. 2005; 102:15545–15550.
 24. Wiśniewski JR, Zougman A, Nagaraj N, Mann M. Universal sample preparation method for proteome analysis. Nat Methods. 2009;6(5):359–362. doi:10.1038/nmeth.1322.
 25. Rodríguez-Piñeiro AM, Bergström JH, Ermund A, Gustafsson JK, Schütte A, Johansson MEV, Hansson GC. Studies of mucus in mouse stomach, small intestine, and colon. II. Gastrointestinal mucus proteome reveals Muc2 and Muc5ac accompanied by a set of core proteins. Am J Physiol Gastrointest Liver Physiol. 2013;305(5):G348–G356. doi:10.1152/ajpgi.00047.2013.
 26. Fritsche K, Boccellato F, Schlaermann P, Koeppl M, Denecke C, Link A, Malfertheiner P, Gut I, Meyer TF, Berger H, et al. DNA methylation in human gastric epithelial cells defines regional identity without restricting lineage plasticity. Clin Epigenet. 2022;14(1):193. doi:10.1186/s13148-022-01406-4.
 27. Moss SF, Legon S, Davies J, Calam J. Cytokine gene expression in *Helicobacter pylori* associated antral gastritis. Gut. 1994;35(11):1567–1570. doi:10.1136/gut.35.11.1567.
 28. Peek Jr RM, Miller GG, Perez-Perez GI, Zhao X, Atherton JC, Blaser MJ. Heightened inflammatory response and cytokine expression in vivo to cagA+ *Helicobacter pylori* strains. Lab Invest. 1995;73(6):760–770.
 29. Yamamoto T, Kita M, Ohno T, Iwakura Y, Sekikawa K, Imanishi J. Role of tumor necrosis factor-alpha and interferon-gamma in *Helicobacter pylori* infection. Microbiol Immunol. 2004;48(9):647–654. doi:10.1111/j.1348-0421.2004.tb03474.x.
 30. Bauer B, Pang E, Holland C, Kessler M, Bartfeld S, Meyer T. The *Helicobacter pylori* virulence effector CagA abrogates human β -defensin 3 expression via inactivation of EGFR signaling. Cell Host Microbe. 2012;11(6):576–586. doi:10.1016/j.chom.2012.04.013.
 31. Pero R, Coretti L, Nigro E, Lembo F, Laneri S, Lombardo B, Daniele A, Scudiero O. β -defensins in the fight against *Helicobacter pylori*. Molecules. 2017;22(3):424. doi:10.3390/molecules22030424.
 32. Sinha M, Kaushik S, Kaur P, Sharma S, Singh TP. Antimicrobial lactoferrin peptides: the hidden players in the protective function of a multifunctional protein. Int J Pept. 2013;2013:390230. doi:10.1155/2013/390230.
 33. Moschen AR, Gerner R, Wang J, Klepsch V, Adolph T, Reider S, Hackl H, Pfister A, Schilling J, Moser P, et al. Lipocalin 2 protects from inflammation and tumorigenesis associated with gut microbiota alterations. Cell Host Microbe. 2016;19(4):455–469. doi:10.1016/j.chom.2016.03.007.
 34. Pasupuleti M, Walse B, Nordahl EA, Mörgelin M, Malmsten M, Schmidtchen A. Preservation of antimicrobial properties of complement peptide C3a, from invertebrates to humans. J Biol Chem. 2007;282(4):2520–2528. doi:10.1074/jbc.M607848200.
 35. Reid-Yu SA, Tuinema BR, Small CN, Xing L, Coombes BK, Gaynor EC. CXCL9 contributes to antimicrobial protection of the gut during *Citrobacter rodentium* infection independent of chemokine-receptor signaling. PLOS Pathog. 2015;11(2):e1004648. doi:10.1371/journal.ppat.1004648.
 36. Hunter HN, Demcoe AR, Jenssen H, Gutteberg TJ, Vogel HJ. Human lactoferricin is partially folded in aqueous solution and is better stabilized in a membrane mimetic solvent. Antimicrob Agents Chemother. 2005;49(8):3387–3395. doi:10.1128/AAC.49.8.3387-3395.2005.
 37. Howitt MR, Lee JY, Lertsethtakarn P, Vogelmann R, Joubert L-M, Ottemann KM, Amieva MR. ChePep controls *Helicobacter pylori* infection of the gastric glands and chemotaxis in the Epsilonproteobacteria. mBio. 2011;2(4). doi:10.1128/mBio.00098-11.
 38. Sigal M, Rothenberg ME, Logan CY, Lee JY, Honaker RW, Cooper RL, Passarelli B, Camorlinga M, Bouley DM, Alvarez G, et al. *Helicobacter pylori* activates and expands Lgr5(+) stem cells through direct colonization of the gastric glands. Gastroenterology. 2015;148(7):1392–1404.e1321. doi:10.1053/j.gastro.2015.02.049.
 39. Larsson JM, Karlsson H, Sjövall H, Hansson GC. A complex, but uniform O-glycosylation of the human MUC2 mucin from colonic biopsies analyzed by nanoLC/MSn. Glycobiology. 2009;19(7):756–766. doi:10.1093/glycob/cwp048.
 40. Gustafsson JK, Sjövall H, Hansson GC. Ex vivo measurements of mucus secretion by colon explants. Methods Mol Biol. 2012;842:237–243.
 41. Helena Macedo M, Baião A, Pinto S, Barros AS, Almeida H, Almeida A, Das Neves J, Sarmiento B. Mucus-

- producing 3D cell culture models. *Adv Drug Deliv Rev.* 2021;178:113993. doi:10.1016/j.addr.2021.113993.
42. Dolan B, Ermund A, Martinez-Abad B, Johansson MEV, Hansson GC. Clearance of small intestinal crypts involves goblet cell mucus secretion by intracellular granule rupture and enterocyte ion transport. *Sci Signaling.* 2022;15(752):eab15848. doi:10.1126/scisignal.abl5848.
 43. Liu L, Saitz-Rojas W, Smith R, Gonyar L, In JG, Kovbasnjuk O, Zachos NC, Donowitz M, Nataro JP, Ruiz-Perez F, et al. Mucus layer modeling of human colonoids during infection with enteroaggregative *E. coli*. *Sci Rep.* 2020;10(1):10533. doi:10.1038/s41598-020-67104-4.
 44. Dupont A, Heinbockel L, Brandenburg K, Hornef MW. Antimicrobial peptides and the enteric mucus layer act in concert to protect the intestinal mucosa. *Gut Microbes.* 2014;5(6):761–765. doi:10.4161/19490976.2014.972238.
 45. Antoni L, Nuding S, Weller D, Gersemann M, Ott G, Wehkamp J, Stange EF. Human colonic mucus is a reservoir for antimicrobial peptides. *J Crohn's Colitis.* 2013;7(12):e652–e664. doi:10.1016/j.crohns.2013.05.006.
 46. Geitani R, Moubareck CA, Xu Z, Karam Sarkis D, Touqui L. Expression and roles of antimicrobial peptides in innate defense of airway mucosa: potential implication in cystic fibrosis. *Front Immunol.* 2020;11:1198. doi:10.3389/fimmu.2020.01198.
 47. Hennart PF, Brasseur DJ, Delogne-Desnoeck JB, Dramaix MM, Robyn CE. Lysozyme, lactoferrin, and secretory immunoglobulin A content in breast milk: influence of duration of lactation, nutrition status, prolactin status, and parity of mother. *Am J Clin Nutr.* 1991;53(1):32–39. doi:10.1093/ajcn/53.1.32.
 48. Masson PL, Heremans JF. Lactoferrin in milk from different species. *Comp Biochem Physiol B.* 1971;39(1):119–129. doi:10.1016/0305-0491(71)90258-6.
 49. González-Chávez SA, Arévalo-Gallegos S, Rascón-Cruz Q. Lactoferrin: structure, function and applications. *Int J Antimicrob Agents.* 2009;33(4):301–308. doi:10.1016/j.ijantimicag.2008.07.020.
 50. Vega-Bautista A, de la Garza M, Carrero JC, Campos-Rodríguez R, Godínez-Victoria M, Drago-Serrano ME. The impact of lactoferrin on the growth of intestinal inhabitant bacteria. *Int J Mol Sci.* 2019;20(19):4707. doi:10.3390/ijms20194707.
 51. van Berkel PH, Geerts EJM, VAN Veen AH, Mericskay M, DE Boer AH, Nuijens HJ. N-terminal stretch Arg2, Arg3, Arg4 and Arg5 of human lactoferrin is essential for binding to heparin, bacterial lipopolysaccharide, human lysozyme and DNA. *Biochemical J.* 1997;328(1):145–151. doi:10.1042/bj3280145 .
 52. Miehlike S, Reddy R, Osato MS, Ward PP, Conneely OM, Graham DY. Direct activity of recombinant human lactoferrin against *Helicobacter pylori*. *J Clin Microbiol.* 1996;34(10):2593–2594. doi:10.1128/jcm.34.10.2593-2594.1996.
 53. Yuan Y, Wu Q, Cheng G, Liu X, Liu S, Luo J, Zhang A, Bian LI, Chen J, Lv J, et al. Recombinant human lactoferrin enhances the efficacy of triple therapy in mice infected with *Helicobacter pylori*. *Int J Mol Med.* 2015;36(2):363–368. doi:10.3892/ijmm.2015.2251.
 54. Guo BX, Wang Q-Q, Li J-H, Gan Z-S, Zhang X-F, Wang Y-Z, Du H-H. Lipocalin 2 regulates intestine bacterial survival by interplaying with siderophore in a weaned piglet model of *Escherichia coli* infection. *Oncotarget.* 2017;8(39):65386–65396. doi:10.18632/oncotarget.18528.
 55. Zhang XJ, Zhong Y-Q, Ma Z-Y, Hu Y-Z, Su J-G, Zhang Y-A. Insights into the antibacterial properties of complement peptides C3a, C4a, and C5a across vertebrates. *J Immunol (Baltimore, Md. : 2022);*1950(12):2330–2340. doi:10.4049/jimmunol.2101019.
 56. Crawford MA, Fisher DJ, Leung LM, Lomonaco S, Lascols C, Cannatelli A, Giani T, Rossolini GM, Doi Y, Goodlett DR, et al. CXC chemokines exhibit bactericidal activity against multidrug-resistant gram-negative pathogens. *mBio.* 2017;8(6). doi:10.1128/mBio.01549-17.
 57. Liu T, Zhang L, Joo D, Sun S-C. Nf-κB signaling in inflammation. *Signal Transduct And Targeted Ther.* 2017;2(1):17023. doi:10.1038/sigtrans.2017.23.
 58. Keates S, Hitti YS, Upton M, Kelly CP. *Helicobacter pylori* infection activates nf-kappa B in gastric epithelial cells. *Gastroenterology.* 1997;113(4):1099–1109. doi:10.1053/gast.1997.v113.pm9322504.
 59. Zimmermann S, Pfannkuch L, Al-Zeer MA, Bartfeld S, Koch M, Liu J, Rechner C, Soerensen M, Sokolova O, Zamyatina A, et al. ALPK1- and TIFA-Dependent innate immune response triggered by the *Helicobacter pylori* type IV secretion system. *Cell Rep.* 2017;20(10):2384–2395. doi:10.1016/j.celrep.2017.08.039.
 60. Pfannkuch L, Hurwitz R, Trauisen J, Sigulla J, Poeschke M, Matzner L, Kosma P, Schmid M, Meyer TF. ADP heptose, a novel pathogen-associated molecular pattern identified in *Helicobacter pylori*. *FASEB J.* 2019;33(8):9087–9099. doi:10.1096/fj.201802555R.
 61. Maubach G, Vieth M, Boccillato F, Naumann M. *Helicobacter pylori*-induced nf-κB: trailblazer for gastric pathophysiology. *Trends Mol Med.* 2022;28(3):210–222. doi:10.1016/j.molmed.2021.12.005.